Topological and kinetic aspects of phospholipids in blood coagulation

(3)

TOPOLOGICAL AND KINETIC ASPECTS OF PHOSPHOLIPIDS IN BLOOD COAGULATION

R.F.A.ZWAAL, J.ROSING, G.TANS, E.M.BEVERS AND H.C.HEMKER

Department of Biochemistry, State University of Limburg, Maastricht, The Netherlands

INTRODUCTION

It has been recognised for many years that the procoagulant activity of certain phospholipids is brought about by providing a catalytic surface on which various coagulation factors adsorb in order to acquire efficient interaction. The catalytic action of phospholipids plays a crucial role in at least three transitory stages of the coagulation cascade. The best known example is the conversion of prothrombin into thrombin by the prothrombinase complex, which requires at least four components in addition to substrate prothrombin, the proteins factor X_a and factor V_a, Ca^{2+} and phospholipids (see ref. 1 for a recent review). Of these components, only factor X_a is able to convert prothrombin into thrombin by limited proteolysis, but its individual action is considerably slower than that of the complete prothrombinase complex. It is well known that the clot-promoting activity of phospholipids in vitro is not attributable to a certain phospholipid class, but to a specific negative charge of the phospholipid surface. In particular, negatively charged phospholipids such as phosphatidylserine, phosphatidylinosithol or phosphatidylglycerol (when properly diluted with neutral phospholipids like phosphatidylcholine or phosphatidylethanolamine) exhibit marked activation of the coagulation process. In situ, the platelet plasma membrane presumably provides the functional phospholipid-water interface required for the prothrombinase complex. This procoagulant activity, commonly referred to as platelet factor 3, is normally unavailable in the circulating intact platelet but becomes unmasked when platelets are activated by specific stimulants.

This article deals with two different but complementary concepts of the role of phospholipids in coagulation, namely the mechanism by which platelets generate a procoagulant phospholipid surface upon platelet activation, and the role of these phospholipids in the kinetics of thrombin formation from prothrombin.

LOCALISATION OF (PROCOAGULANT) PHOSPHOLIPIDS IN PLATELET PLASMA MEMBRANES

In order to gain insight in the mechanism of platelet factor 3 generation, it is required to know where the (procoagulant) phospholipids are localised in

the non-activated intact platelet. An initial approach to this problem was carried out by Schick et al [2]. On the basis of labelling experiments of intact and lysed platelets with the non-permeable reagent trinitrobenzene-sulphonate, they proposed that all phosphatidylserine and the major fraction of phosphatidylethanolamine are located at the inside of the platelet membrane. As an extension of previous work on the action of highly purified phospholipases on erythrocytes [3,4], in which it was shown that these enzymes are useful tools in revealing the phospholipid distribution between both sides of the membrane, the action of a number of phospholipases on intact and lysed platelets was studied [5].

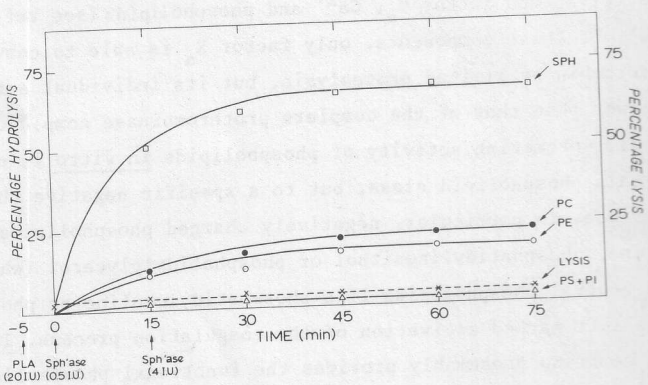

Fig. 1. Non-lytic degradation of pig platelet phospholipids during prolonged incubations with N.naja phospholipase A_2 (PLA) and S.aureus sphingomyelinase (Sph'ase). Indicated number of international units (IU) was used per 100 mg platelets (wet weight). Abbreviations: SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

As depicted in Fig. 1, extensive degradation of the phospholipids present at the pig platelet surface was obtained by the successive action of phospholipase A_2 (Naja naja) and sphingomyelinase (Staphylococcus areus) without

significant lysis of the cells. The energetic metabolism of the cells was maintained at a sufficient level by 25 mM glucose, and under the conditions used no release of N-acetyl-β-glucosaminidase, β-glucuronidase, nucleotides or serotonin occurred. Apparently, sphingomyelin degradation was most extensive whereas smaller amounts of phosphatidylcholine and phosphatidylethanolamine were hydrolysed. Hydrolysis of phosphatidylserine and phosphatidylinositol was very limited. Similar results were obtained with human platelets, the main difference being a somewhat lower availability of phosphatidylethanolamine to phospholipase attack. On the other hand, incubation of lysed platelets with the two phospholipases resulted in a nearly complete degradation of all phospholipid classes. The observed non-lytic degradation (maximally corresponding to 25% and 29% of human and pig platelet phospholipids, respectively) is considered to occur at the outside of the plasma membrane, thus suggesting a non-random phospholipid distribution in this membrane. The phospholipid fraction which is not degraded represents constituents from the cytoplasmic surface of plasma membranes as well as from intracellular membranes. In order to calculate the phospholipid distribution over the plasma membrane, the ratio of the phospholipids between plasma and intracellular membranes has to be determined. Since it is not possible yet to isolate platelet plasma membranes in a quantitative way, this ratio was determined in an indirect manner by analyzing plasma membranes from sphingomyelinase treated and control cells (for details consult ref. 5). The isolation of plasma membranes from cells subjected to non-lytic treatment by sphingomyelinase yielded preparations with similar activities of marker enzymes as membranes obtained from control cells. This approach allowed to estimate that approx. 63% of the platelet phospholipids are located in the plasma membrane. The distribution of phospholipids between the exterior and interior surface of the plasma membrane has been deduced by using this value and comparing the hydrolytic action of both phospholipases on intact and lysed cells. Table I gives the phospholipid composition of the pig platelet membrane and the calculated values of the percentage hydrolysis of the phospholipids in the plasma membrane by phospholipases under non-lytic conditions. The 29% degradation of total phospholipid in intact pig platelets corresponds to 46% hydrolysis of the plasma membrane phospholipids which fraction is considered to represent the outer half of the lipid bilayer. The asymmetric distribution of phospholipids between the interior and exterior half of the pig platelet plasma membrane is shown schematically in Fig. 2, and this pattern appears to be very similar to that of the human platelet - and the human erythrocyte plasma membrane.

TABLE I

NON LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 AND SPHINGOMYELINASE C IN THE PIG PLATELET MEMBRANE

Phospholipid composition is the mean of 5 experiments (S.D.)

	Phospholipid composition: % of total phospholipid	Phospholipid degradation	
		% of total phospholipid	% of phospholipid class
Sphingomyelin	26.1 ± 2.3	23.7	91
Phosphatidylcholine	30.4 ± 2.0	12.2	40
Phosphatidylserine + phosphatidylinositol	16.1 ± 1.6	1.0	6
Phosphatidylethanolamine	27.3 ± 2.3	9.2	34
Total phospholipid	100	46.1	

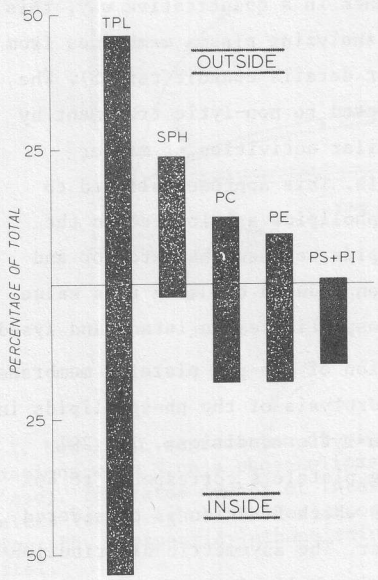

Fig. 2. Asymmetric distribution of phospholipids in the pig platelet plasma membrane. Abbrevations: TPL, total phospholipids. For others, consult legend to Fig. 1.

In all these membranes, phosphatidylserine is nearly exclusively located on the cytoplasmic surface, whereas the outer monolayer of the membrane consists of neutral phospholipids, particularly sphingomyelin. The other two major phospholipid classes, phosphatidylcholine and phosphatidylethanolamine, are present on both membrane sides in a ratio which varies with the origin of the membrane.

The observed absence of negatively charged phospholipids at the exterior surface of platelets may explain why circulating platelets display no procoagulant activity, at the same time suggesting that during platelet activation the interior layer of phospholipids of the plasma membrane becomes available to act as an anchoring surface to interacting coagulation factors. Therefore, the clot-promoting activity of liposomes with the same phospholipid composition as present in the outer and inner layers of pig platelet membranes and human erythrocyte membranes was investigated [6,7]. For this purpose, a one-stage prothrombinase assay was used in which factor X was activated by factor X activating enzyme from Russell's viper venom (RVV-X) in the presence of $CaCl_2$, prior to addition of different amounts of phospholipids. The coagulation time was measured as a function of the phospholipid concentration. As shown in Fig. 3, liposomes having the same composition as the outer membrane layer of either platelets or erythrocytes did not shorten the clotting time of human platelet-poor plasma in this assay. In contrast, liposomes simulating the phospholipid composition found in the cytoplasmic surfaces of these blood cell membranes considerably reduced the clotting time. It was also shown that different preparations of erythrocyte membranes exposing only the exterior surface (intact cells and resealed ghosts) had no procoagulant activity, whereas membrane preparations with the cytoplasmic surface exposed (non-sealed ghosts and sealed inside-out vesicles) produced a strong reduction in clotting time. These results strongly suggest that only the phospholipids at the membrane interior are active in coagulation, and that the outer surface of blood cells is devoid of a phospholipid matrix with procoagulant activity.

GENERATION OF PROCOAGULANT PHOSPHOLIPIDS FOLLOWING PLATELET ACTIVATION

Detection of the generation of platelet factor 3 activity upon platelet activation requires at least some form of quantitation. However, the procoagulant activity of phospholipids and membranes, measured in a one-stage prothrombinase assay, is dependent on a number of variables.

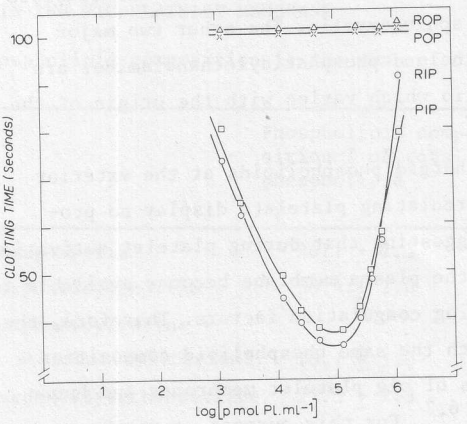

Fig.3. Effect of liposomes on clotting time using a one-stage prothrombinase assay. Liposomes simulate inner and outer half of red cell and platelet plasma membrane. Abbreviations: ROP, red cell membrane outer phospholipids; RIP, red cell membrane inner phospholipids; POP, platelet plasma membrane outer phospholipids; PIP, platelet plasma membrane inner phospholipids.

(i) The reduction of the clotting time appears to be a function of the procoagulant phospholipid concentration (compare Fig.3). The appearance of an optimal lipid concentration where maximal procoagulant activity occurs is thought to result from the balancing effect of the density of bound clotting factors and the lateral diffusion rates of these proteins over the surface [8,9]. At low lipid concentrations the density will be maximal. The more phospholipid surface is provided the more prothrombinase complexes will be formed and as a result the clotting time becomes shorter. However, at higher lipid concentrations the density of the bound proteins will decrease and so will their collision chance. This effect tends to prolong the clotting time. Therefore, platelet factor 3 assays should be performed at low lipid concentrations where the amount of available lipid surface is rate limiting; if not, a smaller amount of lipid might give rise to a shorter coagulation time.

(ii) Even at the same (rate-limiting) phospholipid concentration, the procoagulant activity is a function of the ratio of negatively charged phospholipid to neutral phospholipid. For example, with mixtures of synthetic phosphatidylserine and phosphatidylcholine (above the phase transition) maximal procoagulant activity was observed between 30 and 70 mole% of phosphatidylserine,

whereas the activity declined at higher and lower phosphatidylserine mole-fractions [9]. If platelet factor 3 generation involves a gradual appearance of increasing amounts of phosphatidylserine at the outer surface, it cannot be distinguished from a gradual exposure of cytoplasmic surface (such as occurs during lysis) when only a clotting assay is used. Therefore, lysis should be controlled and its contribution to reduction of the clotting time measured.
(iii) Different negatively charged phospholipids have been shown to exhibit different clot-promoting activities (see for a recent review ref. 7). In as much as this concerns platelet phospholipids, it can be imagined that exposure of a certain amount of phosphatidylinositol has a different clot-promoting activity as a same amount of phosphatidylserine. We have recently shown [10] that a ternary mixture of equimolar amounts of dioleoyl-phosphatidylcholine, dioleoyl-phosphatidylserine and cholesterol is indistinguishable from extracted platelet lipids in the one-stage prothrombinase assay. Replacing phosphatidyl-serine by phosphatidylinositol gave a considerable reduction in clot-promoting activity.
(iv) Platelets liberate f V (or V_a) upon lysis or activation [11]. This activity has been related to the f X_a receptor on activated platelets, which after binding of X_a and prothrombin might result in considerable thrombin production [12,13,14]. Nevertheless, lysed platelets appeared to have the same procoagulant activity as extracted phospholipids in the one-stage prothrombinase assay as long as the lipid concentration used is rate limiting [10]. However, at higher lipid concentrations f V (or V_a) was found to become rate limiting with a resultant higher procoagulant activity of lysed platelets as compared to their extracted lipids.

For all practical purposes, 100% platelet factor 3 activity can at best be defined as the clot-promoting activity of completely lysed platelets using lipid concentrations which are rate limiting. Different degrees of lysis can be obtained by sonicating platelets for various time periods and can be quantitated by measuring the leakage of the cytoplasmic lactate dehydrogenase (LDH). As shown in Fig. 4, a double logarithmic plot of clotting time versus percentage of LDH leakage yields a straight line between 0.1 and 100% lysis; i.e. a strict correlation can be obtained between procoagulant activity and amount of cytoplasmic surface exposed. Identical results were obtained when 100% lysed platelets are diluted in order to reduce the absolute amount of cytoplasmic surface in the clotting assay.

In vitro handling of platelets, especially during platelet activation by certain stimulants, is inevitably accompanied by a few percent of lysis [15].

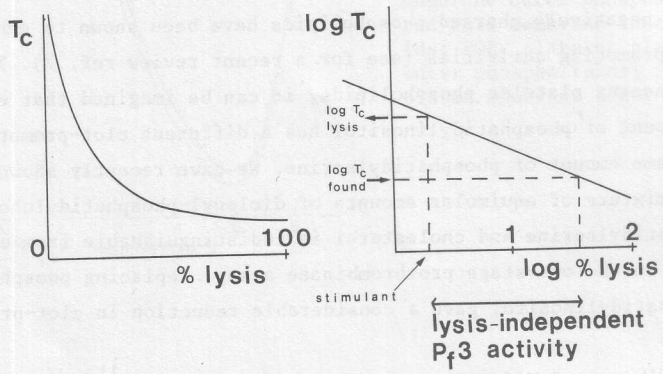

Fig. 4. Determination of lysis-independent platelet factor 3 (Pf 3) activity. Clotting time (T_c) of platelets was measured in a one-stage prothrombinase assay as a function of platelet lysis (left panel) and plotted double logarithmically (right panel). As indicated in the right panel, a certain platelet stimulant produces a shorter clotting time (T_c found) than can be explained on the basis of lysis alone (T_c lysis). Percentage of lysis-independent platelet factor 3 is then expressed as the extra amount of lysis (or the extra amount of cytoplasmic surface) that would have been required to produce the observed clotting time.

This will produce a considerable reduction in clotting time (compare Fig. 4) due to exposure of the cytoplasmic surface which contains procoagulant phospholipids. In as much as this is considered to represent platelet factor 3 activity it can simply be explained by lysis, but it remains to be established whether lysis occurs to any significant extent when platelets become activated in vivo. In a series of experiments human platelets were activated by a variety of platelet stimulants and aggregation, serotonin release and percentage of lysis measured by LDH liberation was detected. The procoagulant activity of these platelet preparations was measured in the one-stage prothrombinase assay, and corrected for the coagulant activity which can be accounted for by the limited lysis of the cells (Table II). Lysis-independent platelet factor 3 activity is expressed as the percentage of lysis that would be required to produce the same reduction in clotting time minus the actual percentage of lysis caused by the various

TABLE II

PLATELET FACTOR 3 ACTIVITY

Platelet stimulant	Aggregation	% 5 HT release	% lysis (LDH)	T_c lysis (sec)	T_c found (sec)	% Pf 3 (lysis inde-pendent)	% platelet PL exposed to phos-pholipases PE	PS
GFP (no additions)	–	0	0.1	112	114	0	12	1
3 sec sonication	–	N.D.	1.0	66	66	0	N.D.	N.D.
3 min sonication	–	N.D.	100	23.4	23.4	0	N.D.	N.D.
ADP (10 μM)	+	0.5	1.1	64.5	64	0.1	N.D.	N.D.
Collagen (10 μg/ml)	+	45	1.8	57	49	2	18	1
Thrombin (0.1 U/ml)	+	58	1.7	59	49	2	20	3
A 23187 (3 μM)	+	96	1.7	59	27.5	41	85-90	50-65
A 23187 + EDTA	–	15	1.8	57	56	0.1	14	2
A 23187 + thrombin	+	88	0.9	68	23	104	85-90	50-65
Collagen + thrombin	+	82	0.6	74	35	15	55-60	50-60

Abbrevations: 5 HT, serotonin; T_c, clotting time; Pf 3, platelet factor 3; PL, phospholipid; PE, phosphatidyl-ethanolamine; PS, phosphatidylserine; GFP, gelfiltered platelets; N.D., not determined. Consult also Fig. 4.

treatments, read from the calibration curve shown in Fig. 4. It appeared that lysis-independent platelet factor 3 activity was only generated by the Ca-ionophore A 23187 (the effect of which was potentiated by thrombin) and by the combined action of collagen and thrombin, provided that Ca^{2+} was present in the external medium. The coagulant activity induced by other combinations or by the separate action of collagen, thrombin or ADP, is caused by the small amount of lysis accompanying platelet activation. When (aggregated) platelets activated with ionophore or with a combination of collagen and thrombin were centrifuged at 5000 x g for 5 min, more than 85% of the lysis-independent platelet factor 3 activity was recovered after resuspending the pellet in buffer. This indicates that the majority of this activity is associated with activated (non-lysed) platelets and is not released as a separate entity into the medium.

When activated platelets were treated with N.naja phospholipase A_2 either alone or in combination with S.aureus sphingomyelinase, lysis mounted to 10-15%. Nevertheless, only in those preparations which exhibited considerable lysis-independent platelet factor 3 activity, 50-65% of phosphatidylserine and some 90% of phosphatidylethanolamine were found to be degraded. It is emphasized that this cannot be explained by the secretory event accompanying platelet release reaction which is thought to involve fusion of intracellular membranes with the plasma membrane. The individual actions of collagen or thrombin produce a considerable release reaction without lysis-independent platelet factor 3 activity or exposure of any significant amount of phosphatidylserine to externally added phospholipases. It can be speculated that the non-lytic exposure of phosphatidylserine at the external surface of platelets, following activation by A 23187 or by the combined action of collagen and thrombin, is brought about by a translocation of phosphatidylserine through the platelet plasma membrane. Although this process, commonly referred to as phospholipid flip-flop, is very slow in sonicated lipid vesicles [16], it has been shown that half-times of the flip-flop rate of dioleoylphosphatidylcholine in vesicles containing integral membrane proteins is in the order of 1 h or less [17]. Moreover, it has been established with different methods that flip-flop rates of phosphatidylcholine in erythrocyte membranes have a half time varying from 2-6 h [18,19,20]. The phospholipid distribution over the platelet plasma membrane following activation by the combined action of collagen plus thrombin is schematically depicted in Fig. 5. Similar distribution patterns are observed after activation by the Ca-ionophore A 23187. It is suggested that these activations trigger a (membrane protein) dependent flip-flop mechanism resulting

in exposure of procoagulant phospholipids (particularly phosphatidylserine) at the outer membrane surface.

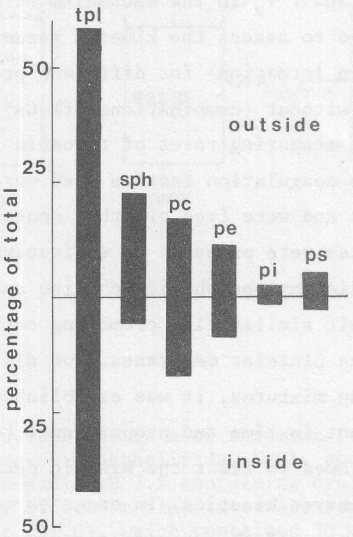

Fig. 5. Asymmetric distribution of phospholipids in the cell membrane after activation of platelets by the combined action of collagen + thrombin. Similar distributions are observed upon platelet activation by the action of the Ca-ionophore A 23187. Abbrevations: consult legends to Figs. 1 and 2.

KINETIC ASPECTS OF PROCOAGULANT PHOSPHOLIPIDS IN THE PROTHROMBINASE COMPLEX

Generation of a procoagulant phospholipid matrix by activated platelets strongly accelerates thrombin production, provided that clotting factors in the initial stages of the coagulation pathway(s) are also activated. Not much is known,however, about the mechanism responsible for the enhancement of the rate of prothrombin activation by phospholipids and f V_a. The observed increase in the relative rate of thrombin formation upon addition of phospho-

lipids and/or factor V_a [22,23], does not provide insight in their mode of action in prothrombin activation, since these experiments were done with single concentrations of prothrombin and of the components of the prothrombinase complex. Moreover, it is known from the work by Esmon et al [21] that no alterations in orders and sites of bond cleavage occur when the prothrombin molecule is activated by different combinations of the components of the prothrombinase complex.

In order to explain the observed rate enhancement and to get more insight in the role of phospholipid and f V_a in the mechanism of prothrombin activation, an assay system was developed to assess the kinetic parameters (K_m for prothrombin and V_{max} of thrombin formation) for different prothrombin activating mixtures, i.e. f X_a with or without (combinations of) Ca^{2+}, f V_a and/or phospholipids [24]. The method for measuring rates of thrombin formation is schematically depicted in Fig. 6. The coagulation factors used were extensively purified using established procedures and were free of other contaminating clotting factors. Phospholipid vesicles were prepared by sonicating an equimolar mixture of highly purified dioleoylphosphatidylcholine and dioleoylphosphatidylserine. These vesicles exhibit similar clot promoting activity in the one-stage prothrombinase assay as platelet membranes. For all different compositions of the prothrombin activating mixtures, it was established that the rate of thrombin formation is constant in time and proportional to the amount of f X_a present. Thus it is allowed to treat the kinetic data according to the Michaelis-Menten model for enzyme kinetics. In order to compare kinetic constants obtained in the presence and absence of accessory components, rates of thrombin formation were measured at optimal concentrations of f V_a and Ca^{2+} and were expressed as mol thrombin produced per min per mol f X_a present. Straight Lineweaver-Burk plots were obtained with all different combinations of the prothrombin activating mixture. Examples showing the conversion of prothrombin by f Xa alone and by the complete prothrombinase complex (at two different phospholipid concentrations) are given in Figs. 7 and 8, respectively. The kinetic parameters of thrombin formation for the different activating mixtures are summarized in Table III. In the absence of accessory components prothrombin is a poor substrate for f X_a. The high K_m value (84 μM) indicates that prothrombin has a low affinity for f X_a under these conditions. Important alterations in the kinetic parameters occur, however, when phospholipids and/or f V_a form part of the prothrombinase complex. The role of f V_a is manifested by a 700 fold increase in the V_{max} of thrombin formation, whereas phospholipids cause a profound decrease of the K_m for prothrombin.

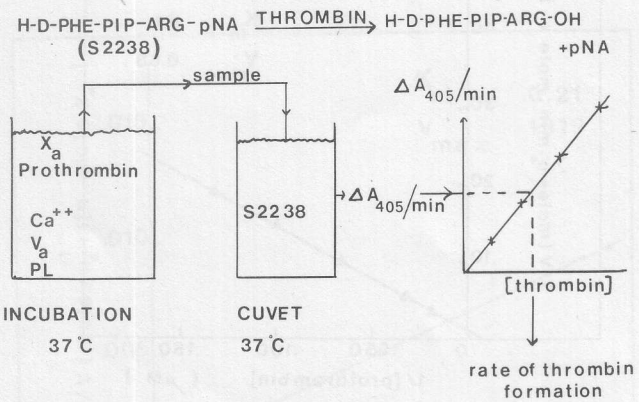

Fig. 6. Method for measuring the rate of thrombin formation. Factor X_a either alone or in the presence of phospholipids, $CaCl_2$ and/or f V_a was incubated for 3-5 min at 37°C in Tris-NaCl, pH 7.5 containing ovalbumin (0.5 mg/ml). Prothrombin was added and after different time intervals samples were taken and added to a thermostated cuvet (37°C), which contained 30 µg soybean-trypsin inhibitor (STI) and 0.47 µmol S 2238 in such an amount of the above buffer that the final volume became 2 ml. The amount of STI inhibits both further conversion of prothrombin and the low amidase activity of f X_a for S 2238; it does not affect the rate of S 2238 conversion by thrombin. From the absorbance change at 405 nm, the amount of thrombin is calculated from a calibration curve made with known amounts of thrombin. The rate of thrombin formation in the incubation mixture is calculated from the amounts of thrombin present in the samples taken from the incubation mixture after different time intervals.

In the presence of phospholipids the K_m drops to values below the plasma concentration of 1.5 µM. From the dramatic alterations of the kinetic parameters we conclude that under physiological conditions significant thrombin formation by f X_a can only occur with the aid of phospholipids and f V_a.

The effects of phospholipids and f V_a on the kinetic parameters explain the earlier observed changes of relative rates of thrombin formation in the presence of accessory components. The question arises which mechanisms are responsible for the alterations in the kinetic constants.

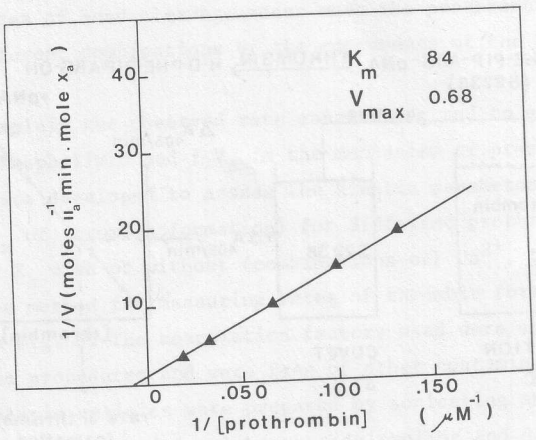

Fig. 7. Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the presence of $CaCl_2$. Thrombin formation at varying concentrations of prothrombin was measured at 37°C in 1 ml of a reaction mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), factor X_a (9.1×10^{-5} μmoles/ml), and 20 mM $CaCl_2$ at pH 7.5. The reaction was started by addition of factor X_a. After $7\frac{1}{2}$ and 15 min samples were taken and assayed for thrombin as described in fig. 6.

TABLE III

KINETIC CONSTANTS OF THROMBIN FORMATION WITH VARIOUS PROTHROMBIN ACTIVATING MIXTURES

Prothrombin activating mixture	K_m for prothrombin	V_{max} mol $II_a min^{-1}$ mol X_a^{-1}
F X_a, $CaCl_2$	84	0.68
F X_a, $CaCl_2$, F V_a	34	373
F X_a, $CaCl_2$, PL (7.5 μM)	0.058	2.25
F X_a, $CaCl_2$, PL (7.5 μM), F V_a	0.21	1919

PL = equimolar mixture of dioleoyl phosphatidylcholine and dioleoylphosphatidylserine.

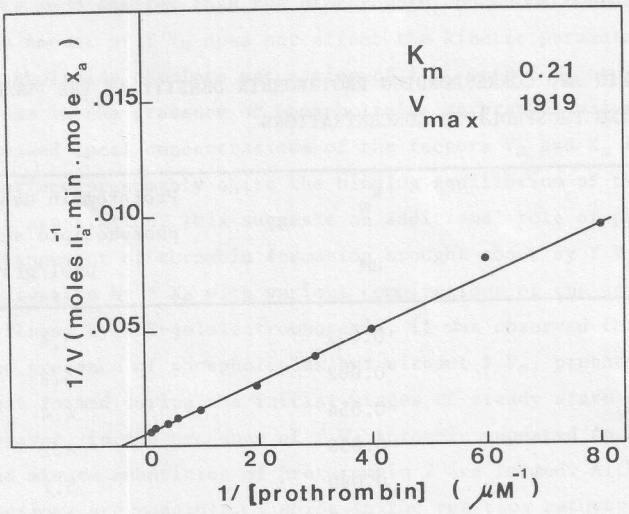

Fig. 8. Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the presence of $CaCl_2$, phospholipid and factor V_a. The experimental details are described in Figs. 6 and 7, except that different concentrations were used: 7.75×10^{-10} µmoles X_a/ml; 7.5 mM $CaCl_2$; 0.9 U f V_a/ml and 7.5 µM phospholipid.

When phospholipids are present, the K_m for prothrombin appeared to be dependent upon the amount of phospholipid added to the incubation mixture. Higher K_m values are found at increasing phospholipid concentrations. As shown in Table IV, the K_m for prothrombin gradually increases from 0.032 µM at 2.6 µM phospholipid to 1.08 µM at a phospholipid concentration of 240 µM. Therefore, a K_m determined in the presence of phospholipids should be regarded as an apparent K_m. This is to be expected since the K_m is expressed in terms of added prothrombin. However, the thrombin formed at the phospholipid surface is in fact generated from prothrombin bound to that surface. Therefore, we calculated the prothrombin density at the phospholipid surface using binding data reported by Nelsestuen and Broderius [25]. For conditions approaching

those of our experiments as close as possible, they reported a dissociation constant of 10^{-7} M for the prothrombin-phospholipid complex and 17 µmoles prothrombin binding sites per gram of phospholipid. The prothrombin density at the phospholipid surface now expressed as µmol prothrombin bound per gram of phospholipid at the K_m measured at different phospholipid concentrations is presented in Table IV.

TABLE IV

K_m FOR PROTHROMBIN AND CORRESPONDING PROTHROMBIN DENSITY AT THE PHOSPHOLIPID SURFACE OF VARYING PHOSPHOLIPID CONCENTRATIONS

Phospholipid µM	K_m µM	Prothrombin density at phospholipid surface µmol/gram
2.6	0.032	3.4
4.0	0.062	5.2
5.3	0.054	4.4
7.5	0.058	4.2
8.0	0.090	5.7
10.5	0.068	4.2
16.0	0.14	6.2
26.3	0.164	5.5
40.0	0.23	5.7
52.6	0.25	5.4
75	0.35	5.1
80	0.46	6.2
105	0.48	5.2
240	1.08	5.4

Although the apparent K_m is increasing with increasing phospholipid concentrations, the prothrombin density at the K_m is independent of the phospholipid concentration. This shows that in the presence of phospholipids the kinetics of thrombin formation is determined by the local prothrombin concentration at the phospholipid surface. At present, no conclusions can be drawn whether the increased local prothrombin concentration fully explains the observed decrease in apparent K_m. A favourable juxtaposing effect of f X_a and prothrombin upon interaction with phospholipids may still contribute to the observed decrease

in K_m. Legitimate conclusions in this connection require knowledge of diffusion constants in solution and at the phospholipid surface, appropriate binding data, and orientation at the phospholipid surface of the proteins involved.

With respect to the mode of action of f V_a it seems plausible to assume that the X_a-V_a complex is the active catalytic unit, since the optimal f V_a concentration is much smaller than the prothrombin concentration, and a further increase in the amount of f V_a does not affect the kinetic parameters. In the absence of phospholipids complete saturation of f X_a with f V_a could not be obtained, whereas in the presence of phospholipids saturation was readily achieved. Increased local concentrations of the factors V_a and X_a at the phospholipid surface presumably shift the binding equilibrium of the two components towards association. This suggests an additional role of phospholipids in the rate enhancement of thrombin formation brought about by f V_a. When prothrombin activation by f X_a with various combinations of the accessory components was followed by SDS-gelelectrophoresis, it was observed that both in the absence and presence of phospholipids but without f V_a, prethrombin 2 is the main product formed during the initial stages of steady state prothrombin activation. However, in the presence of f V_a thrombin appeared to be the main end product and minute quantities of prethrombin 2 are formed. Although more complex explanations are possible, a shift in the reaction pathway from one leading mainly to prethrombin 2 to one leading almost exclusively to thrombin upon addition of f V_a can qualitatively account for the observed increase in V_{max}. The f V_a binding site, located in the fragment 2 region of the prothrombin molecule [21], may well contribute to keeping the initially formed prethrombin 2 molecule with the complex offering it a second chance to be converted with resultant thrombin formation.

CONCLUDING REMARKS

Our observations have important implications for the *in vivo* situation, although it is realized that the conditions in the blood vessel differ in many respects from those in the test tube. Our data point out that during triggering of the coagulation process the platelet plasma membrane becomes activated to expose functionally active phospholipids formerly present at the cytoplasmic surface. As a consequence, the K_m for prothrombin strongly decreases to become markedly smaller than the plasma prothrombin concentration. This results in a considerable increase in apparent velocity of thrombin production and clot formation. The required amount of factor X_a can be provided by factor VII_a action in the presence of tissue thromboplastin during the initial time course of the coagulation process. In fact it has been observed [26] that tissue factor,

which is a lipoprotein, increases the k_{cat} of factor X activation by factor VII_a about 2700 fold and produces a 10 fold decrease in the K_m for factor X. Intrinsic factor X_a formation by factor IX_a can profit from the same phospholipid matrix exposed at the exterior platelet surface, provided that the kinetics of the reaction are comparable to those for prothrombin activation.

REFERENCES

1. Suttie, J.W. and Jackson, C.M. (1977) Physiol.Rev. 57, 1-70.
2. Schick, P.K., Kurica, K.B. and Chacko, G.K. (1976) J.Clin.Invest. 57, 1221-1226.
3. Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D. and van Deenen, L.L.M. (1973) Biochim.Biophys.Acta 323, 178-193.
4. Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and van Deenen, L.L.M. (1975) Biochim.Biophys.Acta 406, 83-96.
5. Chap, H.J., Zwaal, R.F.A. and van Deenen, L.L.M. (1977) Biochim.Biophys. Acta 467, 146-164.
6. Zwaal, R.F.A., Comfurius, P. and van Deenen, L.L.M. (1977) Nature 268, 360-362.
7. Zwaal, R.F.A. (1978) Biochim.Biophys.Acta 515, 163-205.
8. Hemker, H.C. (1975) in Handbook of Hemophilia (Brinkhous, K.M. and Hemker, H.C.,eds) Chapter 3, p. 31-48, Excerpta Medica, Amsterdam.
9. Tans, G., van Zutphen, H., Comfurius, P., Hemker, H.C., and Zwaal, R.F.A. (1979) Eur.J.Biochem. 95, 449-457.
10. van Zutphen, H., Bevers, E.M., Hemker, H.C. and Zwaal, R.F.A. (1979) Brit. J.Haematol., submitted.
11. Østerud, B., Rapaport, S.I. and Lavine, K.K. (1977) Blood 49, 819-834.
12. Miletich, J.P., Jackson, C.M. and Majerus, P.W. (1977) Proc.Natl.Acad. Sci.U.S. 74, 4033-4036.
13. Miletich, J.P., Jackson, C.M. and Majerus, P.W. (1978) J.Biol.Chem. 253, 6908-6916.
14. Miletich, J.P., Majerus, D.W. and Majerus, P.W. (1978) J.Clin.Invest. 62, 824-831.
15. Joist, J.H., Dolezel, G., Lloyd, J.V., Kinlough-Rathbone, R.L. and Mustard, J.F. (1974) J.Lab.Clin.Med. 84, 474-482.
16. Johnson, L.W., Hughes, M.E. and Zilversmit, D.B. (1975) Biochim.Biophys. Acta 375, 176-185.
17. van Zoelen, E.J.J., de Kruijff, B. and van Deenen, L.L.M. (1978) Biochim. Biophys.Acta 508, 97-108.
18. Renooij, W., van Golde, L.M.G., Zwaal, R.F.A. and van Deenen, L.L.M. (1976) Eur.J.Biochem. 61, 53-58.
19. Bloj, B. and Zilversmit, D.B. (1976) Biochemistry 15, 1277-1284.
20. Rousselet, A., Gurthmann, C., Matricon, J., Bienvenue, A. and Devaux, P.F. (1976) Biochim.Biophys.Acta 426, 357-371.
21. Esmon, C.T. and Jackson, C.M. (1974) J.Biol.Chem. 249, 7791-7797.
22. Jobin, F. and Esnouf, M.P. (1967) Biochem.J. 102, 666-674.
23. Esmon, C.T., Owen, W.G. and Jackson, C.M. (1974) J.Biol.Chem. 249, 8045-8047.
24. Rosing, J., Tans, G., Govers-Riemslag, J.W.P., Zwaal, R.F.A. and Hemker, H.C. (1979) J.Biol.Chem., in the press.
25. Nelsestuen, G.L. and Broderius, M. (1977) Biochemistry 16, 4172-4177.
26. Silverberg, S.A., Nemerson, Y. and Zur, M. (1977) J.Biol.Chem. 252, 8481-8488

are determined by the lateral diffusion? Now, if you envision that model, then I think you have an explanation for the effect that you see with the phospholipids. If you envision a totally random model, and I assure you the kinetics that you come up with between an ordered and a random addition of those is very dramatically different, then I think you have some problem with that whole thing. I am just curious as to whether you are even thinking at this time about the way that is assembled, and if you are, which way is it coming down?

Zwaal:

I think we cannot speak in terms of specific binding sites, since in artificial phospholipid bilayer systems, the surface composition is the same no matter from which side it is approached.

Nemerson:

Let me turn the question around. I guess this is somewhat loaded because I have heard the data that Ken Mann presented in London this summer, but you can view this as follows: is the binding constant of prothrombin for phospholipid the same, larger, or smaller than it is for the phospholipid Xa?

Zwaal:

We have not measured accurate binding constants so far with our synthetic phospholipid mixture.

Mann:

I should comment on that. I don't think there is a great deal of difference or inconsistency in most of the conclusions. Our data support the notion that factor Va is driving Xa onto the membrane, and that prothrombin is binding to the membrane principally because of the acidic phospholipid as Dr. Zwaal has discussed. After that event, then what the order of events is I have no idea. I don't think anybody does.

Nemerson:

Are you saying that prothrombin and assembly on the phospholipid is dependent on Xa binding to factor Va?

Mann:

I think, initially, yes. The next step is limited by the lateral diffusion of these components on the phospholipid vesicle or it is limited by the binding constant of prothrombin for the assembled complexes. There are two different processes driven by two different constants.

Nemerson:

I think some of your data argues against the prothrombin binding. As I saw it the K_m changed substantially while binding stays the same. The two are mutually exclusive.

Zwaal:

Well, you measure on an apparent K_m. The K_m you are measuring is the half maximal rate of thrombin formation. That's all you are doing.

Walsh:

One question about the concentrations of the collagen and thrombin required to induce the PS on the outside of the platelet.

Zwaal:

We have used a final concentration of 10 µgm of collagen per milliliter and the thrombin concentration was extremely low, 0.1 u/ml because otherwise it interferes with the coagulation test. We recently noticed that when you use γ-thrombin that it's very effective. I don't think that collagen has anything to do with it. Collagen has a synergistic action together with thrombin. We have the feeling that thrombin alone might do it as well, but then you need a much higher concentration and we cannot use the platelet factor 3 test anymore to screen for platelet factor 3 activity because of the contribution of thrombin.